Combination Therapy for Radiation-Induced Bone Marrow Aplasia in Nonhuman Primates Using Synthokine SC-55494 and Recombinant Human Granulocyte Colony-Stimulating Factor

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Combination cytokine therapy continues to be evaluated in an effort to stimulate multilineage hematopoietic reconstitution after bone marrow myelosuppression. This study evaluated the efficacy of combination therapy with the synthetic interleukin-3 receptor agonist, Synthokine-SC55494, and recombinant methionyl human granulocyte colony-stimulating factor (rhG-CSF) on platelet and neutrophil recovery in nonhuman primates exposed to total body 700 cGy ^{60}Co gamma radiation. After irradiation on day (d) 0, cohorts of animals subcutaneously received single-agent protocols of either human serum albumin (HSA; every day [QD], 15 μg/kg/d, n = 10), Synthokine (twice daily [BID], 100 μg/kg/d, n = 5), rhG-CSF (QD, 10 μg/kg/d, n = 5), or a combination of Synthokine and rhG-CSF (BID, 100 and 10 μg/kg/d, respectively, n = 5) for 23 days beginning on d1. Complete blood counts were monitored for 60 days postirradiation and the durations of neutropenia (absolute neutrophil count <500/μL) and thrombocytopenia (platelet count <20,000/μL) were assessed. Animals were provided clinical support in the form of antibiotics, fresh irradiated whole blood, and fluids. All cytokine protocols significantly ($P < .05$) reduced the duration of thrombocytopenia versus the HSA-treated animals. Only the combination protocol of Synthokine + rhG-CSF and rhG-CSF alone significantly shortened the period of neutropenia ($P < .05$). The combined Synthokine/rhG-CSF protocol significantly improved platelet nadir versus Synthokine alone and HSA controls and neutrophil nadir versus rhG-CSF alone and HSA controls. All cytokine protocols decreased the time to recovery to preirradiation neutrophil and platelet values. The Synthokine/rhG-CSF protocol also reduced the transfusion requirements per treatment group to 0 among 5 animals as compared with 2 among 5 animals for Synthokine alone, 8 among 5 animals for rhG-CSF, and 17 among 10 animals for HSA. These data showed that the combination of Synthokine, SC-55494, and rhG-CSF further decreased the cytopenic periods and nadirs for both platelets and neutrophils relative to Synthokine and rhG-CSF monotherapy and suggest that this combination therapy would be effective against both neutropenia and thrombocytopenia consequent to drug- or radiation-induced myelosuppression.

MYELOSUPPRESSION remains the dose-limiting sequelae after radiation and/or chemotherapy despite the therapeutic utility shown with growth factors. Dose intensification and/or schedule compression will likely extend the obligate periods of cytopenia, particularly those of neutropenia and thrombocytopenia. New cytokines and/or cytokine combinations will be required to decrease the associated increased risks of infection and hemorrhage, which remain the leading causes of morbidity and mortality related to these cytotoxic therapies.

Several cytokine combinations have shown variable efficacy in enhancing hematopoietic recovery in preclinical models of radiation or drug-induced myelosuppression.[1-13] Combination cytokine protocols using the multilineage stimulatory aspect of interleukin-3 (IL-3) in addition to the lineage specificity of IL-6, granulocyte colony-stimulating factor (G-CSF), or granulocyte-macrophage colony-stimulating factor (GM-CSF) have been evaluated in an effort to produce a dual-lineage response in favor of generating both platelets and granulocytes. Preclinical data in primate models of radiation or drug-induced aplasia suggest that the combination of IL-3 with GM-CSF in a concurrent protocol or the use of the IL-3/GM-CSF fusion protein PIXY321 are most effective in the production of platelets and granulocytes,[2-4] whereas the combinations of IL-3 with IL-6 or IL-6 plus G-CSF or GM-CSF are not significantly different from the respective cytokines as monotherapy in production of either platelets or granulocytes.[6-10] In this regard, the IL-6/G-CSF and IL-6/GM-CSF combinations produced a significant dual-lineage response equivalent to their combined monotherapy lineage-specific responses.[6-8]

A modest amount of in vitro and in vivo evidence suggested that the combination of IL-3 and G-CSF would enhance both platelet and neutrophil production. The IL-3/G-CSF combination enhanced both the number and size of megakaryocytic colonies, synergistically or additively increased the number of marrow-derived myeloid-committed progenitor cells, and decreased the time course of murine multilineage and blast cell colony formation.[14-17] Concomitant or sequential protocols, as evaluated in normal animals, elicited a modest additive response with regard to the production of neutrophils and platelets.[18,19] In addition, Aglietta et al[20] recently showed the in vivo priming effect of IL-3 for the action of other cytokines, suggesting that maximum stimulation of granulopoiesis would be achieved by the combination of IL-3 plus G-CSF.

We recently showed the therapeutic efficacy of the synthetic cytokine, (Synthokine) SC55494, a high-affinity human IL-3 receptor ligand, in significantly enhancing platelet recovery and lessening the neutrophil nadir in a nonhuman primate model of radiation-induced myelosuppression.[21] This high-affinity IL-3 receptor ligand had shown greater in vitro multilineage hematopoietic activity on human bone marrow progenitors than native IL-3 while being equivocal

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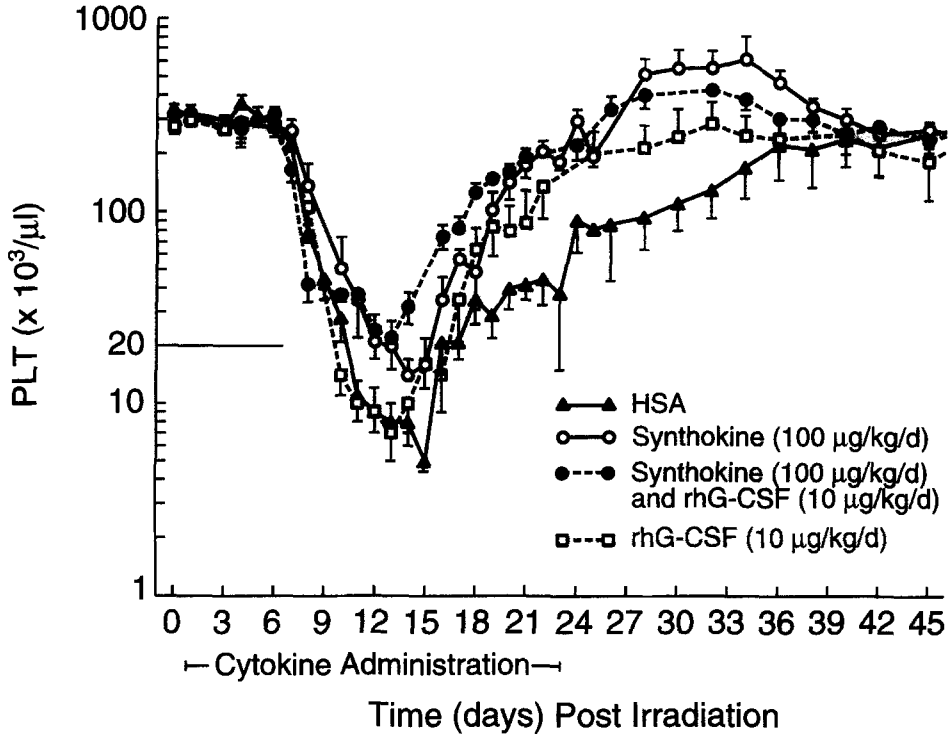

Fig 1. Effects of cytokine administration on peripheral blood platelet counts in irradiated primates. The platelet counts (PLT) were observed in irradiated rhesus primates (700 cGy ^{60}Co) after Synthokine, G-CSF, Synthokine plus G-CSF, or HSA administration as described in the Materials and Methods. Data represent the mean ± SEM of the absolute platelet counts for the cytokine- or HSA-treated animals.

in inflammatory activity.[22] The aim of this study was to evaluate the therapeutic efficacy of concomitant administration of Synthokine SC-55494 plus recombinant methionyl human G-CSF (rhG-CSF) relative to Synthokine and rhG-CSF monotherapy on hematopoietic recovery in a nonhuman primate model of high dose, radiation-induced myelosuppression.

MATERIALS AND METHODS

Animals. Domestic born male rhesus monkeys, *Macaca mulatta* (mean weight, 3.9 ± 0.2 kg), were housed in individual stainless steel cages in conventional holding rooms at the Armed Forces Radiobiology Research Institute (AFRRI) in an animal facility accredited by the American Association for Accreditation of Laboratory Animal Care. Monkeys were provided 10 air changes per hour of 100% fresh air, were conditioned to 72°F ± 2°F with a relative humidity of 50% ± 20%, and were maintained on a 12-hour light/dark full-spectrum light cycle with no twilight. Monkeys were provided with commercial primate chow, supplemented with fresh fruit and tap water ad libitum. Research was conducted according to the principles enunciated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources, National Research Council.[23]

Irradiation. Monkeys placed in an Lucite restraining chair, after a prehabituation period, were bilaterally, total-body irradiated (TBI) with cobalt-60 gamma radiation at the AFRRI Cobalt-60 Facility to a total midline tissue dose of 700 cGy at a dose rate of 40 cGy/min. Dosimetry was performed using a paired 0.5-mL tissue equivalent ionization chamber, whose calibration was traceable to the National Institute of Standards and Technology.

Recombinant cytokines. Synthokine (SC-55494) is an IL-3 receptor agonist and has been described previously.[22] Neupogen (Filgrastim), rhG-CSF, was produced in *Escherichia coli* as a nonglycosylated form of human G-CSF and had a specific activity of 1.0 ± 0.6 × 10^8 U/mg (as measured by a cell mitogenesis assay). The cytokines or the control protein, human serum albumin (HSA; Miles Inc, Cutter Biological, Elkhart, IN), were administered as a 1-mL bolus subcutaneous (SC) injection.

Study design. The animals were randomly assigned to one of three treatment groups composed of a minimum of five animals. Each animal was irradiated on day 0. On day 1, groups of animals received cytokine therapy for 23 consecutive days with one of the following: either rhG-CSF alone (n = 5) or concomitant Synthokine and rhG-CSF (n = 5). The control group (3 concurrent plus 7 contemporary animals) received HSA (n = 10) for the 23-day treatment period. HSA and rhG-CSF were administered once daily (15 or 10 μg/kg, respectively); Synthokine (100 μg/kg) was administered as a divided dose twice daily when administered alone or concomitantly with rhG-CSF. The Synthokine-alone cohort was part of our initial study evaluating dose and protocol. The data have been reported separately and the cohort was not repeated here.[21] The study reported herein was performed in the same time frame as the monotherapy Synthokine study.

Clinical support. An antibiotic regimen was initiated prophylactically when the white blood cell count (WBC) was less than 1,000/μL and was continued daily until the WBC was greater than 1,000/μL for 3 consecutive days. Gentamicin (Lyphomed, Deerfield, IL; 10 mg/d, SC, every day [QD]) and Rocephin (Roche, Nutley, NJ; 250 mg/d, SC, QD) were administered. Fresh, irradiated (1500 cGy ^{60}Co gamma radiation) whole blood (approximately 30 mL/transfusion) from a random donor pool (monkeys of >10 kg body weight) was administered when the platelet (PLT) count was less than 20,000/μL and the hematocrit was less than 18%. Transfusions and antibiotics were required to ensure 100% survival in HSA-treated animals (unpublished results). Animals were observed on a daily basis and inspected for treatment-associated toxicities.

Hematologic evaluations. Peripheral blood was obtained from the saphenous vein to assay complete blood (Model S Plus II; Coulter Electronics, Hialeah, FL) and differential counts (Wright-Giemsa

Table 1. Thrombocytopenia and Neutropenia in Sublethally Irradiated and Cytokine-Treated Rhesus Monkeys: Duration, Nadir, and Time to Recovery

	Duration (d)		Nadir ($\times 10^3/\mu L$)		Time to Recovery (d)	
	THROM	NEUT	Platelet	ANC	Platelet	ANC
HSA	11.9	14.8	5	0.000	40	24
Synthokine	3.5*†	14.1	14*†	0.090*†	24	21
rhG-CSF	7.2*	12.4*	7*	0.004	25	18
Synthokine + rhG-CSF	1.9*†	10.6*	22*†	0.100*†	26	17

Monkeys were whole body irradiated to 700 cGy with ^{60}Co gamma radiation and treated with HSA control protein or Synthokine, rhG-CSF, or concomitant Synthokine plus rhG-CSF according to protocol. Neutropenia (NEUT) is defined as an ANC of less than 500/μL and thrombocytopenia (THROM) is defined as a PLT count of less than 20,000/μL.

* Significantly different from HSA-treated animals ($P < .05$).

† Significantly different from rhG-CSF ($P < .05$).

Stain; Ames Automated Slide Stainer, Elkhart, IN). Baseline levels (BL) were obtained before irradiation. These parameters were monitored for 60 days after irradiation and the durations of neutropenia (absolute neutrophil count [ANC] <500/μL) and thrombocytopenia (PLT < 20,000/μL) were assessed. Whole blood transfusions could have possibly altered the ANC and PLT count; therefore, when determining the durations of neutropenia and thrombocytopenia, ANC and PLT counts had to be maintained for 3 consecutive days above threshold levels after the first increase for a true recovery to be noted.

Statistical analysis. The Normal Scores Test was used to make pairwise comparisons of the durations of neutropenia and thrombocytopenia and to evaluate the statistical significance of the difference between the nadirs. These tests were performed using the software package StatXact (Cytel Software Corp, Cambridge, MA).

RESULTS

Modulation of thrombocytopenia: duration, nadir, recovery time. Thrombocytopenia (PLT < 20,000/μL) was evident in HSA-treated control animals for an average of 11.9 days consequent to 700 cGy TBI (Fig 1 and Table 1). Administration of either Synthokine, rhG-CSF, or the concomitant Synthokine plus rhG-CSF significantly decreased the duration of thrombocytopenia relative to HSA-treated controls to an average of 3.5 days ($P = .001$), 7.2 days ($P = .002$), and 1.9 days ($P < .001$), respectively (Fig 1 and Table 1).[21] The thrombocytopoietic effects of Synthokine alone ($P < .037$) and Synthokine plus rhG-CSF ($P = .006$) were each significantly better than that of rhG-CSF. However, the concomitant Synthokine plus rhG-CSF effect on the duration of thrombocytopenia, although better, was not significantly different from that of Synthokine alone ($P = .179$; 1.9 *v* 3.5 days).

Concomitant administration of Synthokine plus rhG-CSF significantly improved the platelet nadir to 22,000/μL versus 5,000/μL for the HSA-treated controls ($P = .002$), 14,000/μL for Synthokine alone ($P = .004$), and 7,000/μL for rhG-CSF alone ($P = .012$; Fig 1 and Table 1). Synthokine alone ($P = .004$) also significantly improved the platelet nadir relative to controls. Concomitant administration of Synthokine plus rhG-CSF or of Synthokine and rhG-CSF as monotherapy all induced an earlier recovery of platelets to baseline levels (days 26, 24, and 25, respectively, *v* day 40 for controls; Fig 1).

Modulation of neutropenia: duration, nadir, recovery time. The HSA-treated control animals experienced a period of absolute neutropenia and had a mean 14.8-day duration of neutropenia (ANC <500/μL) during which antibiotic support was required (Fig 2 and Table 1). The administration of Synthokine alone did not significantly modify the duration of neutropenia ($P = .20$), although the depth of the nadir was significantly lessened ($P = .002$) relative to HSA-treated controls,[21] whereas rhG-CSF administration significantly lessened the duration of neutropenia to 12.4 days ($P = .028$) but did not modify the neutrophil nadir ($P = .46$). The concomitant administration of Synthokine plus rhG-CSF significantly decreased the duration of neutropenia to 10.6 days ($P = .001$) and improved the ANC nadir to 900/μL ($P = .002$) relative to 0/μL for the HSA-treated controls (Fig 2 and Table 1). Concomitant administration of Synthokine plus rhG-CSF did not significantly decrease the neutropenic duration relative to rhG-CSF alone ($P = .056$) but did significantly decrease the neutropenic duration relative to the Synthokine alone ($P = .004$). The concomitant Synthokine/rhG-CSF protocol also significantly ($P = .004$) lessened the neutrophil nadir associated with rhG-CSF administration (900/μL *v* 4/μL, respectively; Fig 2 and Table 1). All cytokine treatment protocols decreased the time to recovery of neutrophils to preirradiation values.

Modulation of red blood cells (RBCs), transfusion requirements, and toxicities. RBC blood cell counts decreased from an average of $5.75 \times 10^6/\mu L$ over all treatment and control animals to reach similar nadir levels of approximately $3.5 \times 10^6/\mu L$ during the third week after exposure and treatment (Fig 3). Recovery occurred slowly (>60 days) with no significant difference between treatment and control groups. Circulating nucleated RBCs (NRBCs) appeared in HSA-treated animals in the third week after irradiation, with the initial peak at day 24 (Fig 4). Those cohorts treated with Synthokine, rhG-CSF, or Synthokine plus rhG-CSF showed increased circulating NRBCs within the second week, with peak values noted at 18 to 20 days after irradiation and treatment (Fig 4).

Transfusion criteria consisted of a combination of platelet count less than 20,000/μL and a hematocrit less than 18%. Based on these criteria, the combined Synthokine/rhG-CSF–treated animals were transfusion-independent relative to Synthokine alone (2 transfusions among 5 animals), rhG-CSF alone (8 transfusions among 5 animals), and HSA controls (17 transfusions among 10 animals). There was no evidence of clinical bleeding in any particular group of animals. Additionally, there was no evidence of histamine-release–related toxicities or skin reactions.

DISCUSSION

In an effort to increase the therapeutic efficacy of chemotherapy and/or radiotherapy, dose intensification and/or schedule compression will be used. Enhanced recovery from

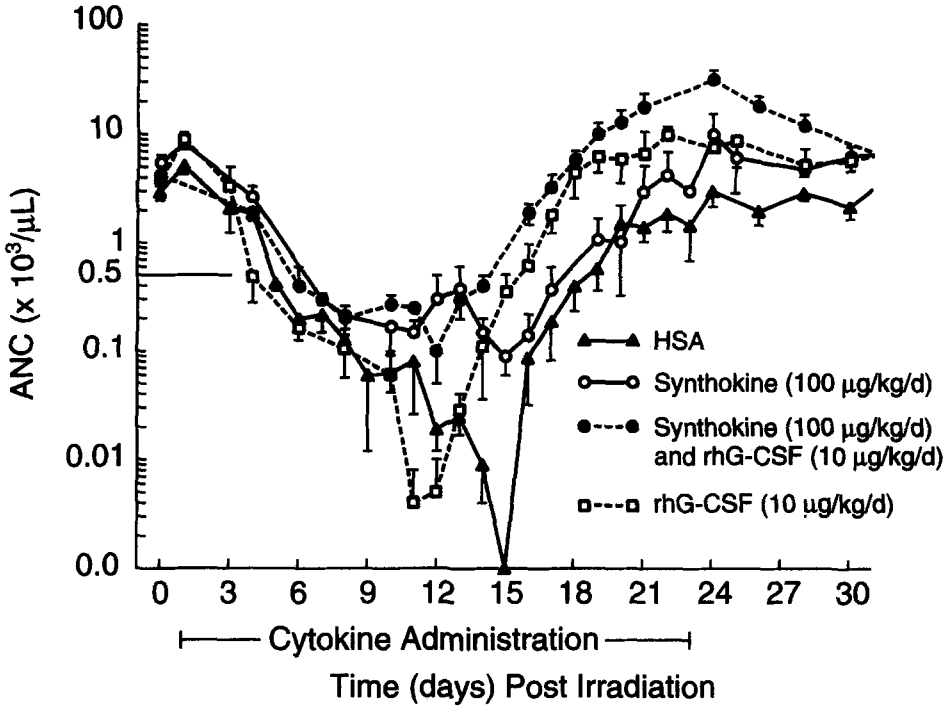

Fig 2. Effects of cytokine administration on peripheral blood ANCs in irradiated primates. The ANCs were observed in irradiated rhesus primates as described in the Materials and Methods. Data represent the mean ± SEM of the ANC for the cytokine- or HSA-treated animals.

the consequent, obligate periods of neutropenia and thrombocytopenia will be required to further reduce the potential infectious and hemorrhagic complications. This study addressed the use of combined cytokine therapy with a synthetic IL-3 receptor agonist, Synthokine-SC-55494, and rhG-CSF in an effort to further increase the production of both platelets and neutrophils in a nonhuman primate model of radiation-induced myelosuppression. The combination protocol was restricted to the concomitant administration of Synthokine plus rhG-CSF relative to the respective controls of Synthokine or rhG-CSF alone.

We showed that concomitant Synthokine/rhG-CSF administration significantly decreased the duration and nadir of thrombocytopenia relative to HSA-control or rhG-CSF–treated cohorts. The Synthokine/rhG-CSF protocol also further decreased, although not significantly, the duration of thrombocytopenia noted for Synthokine alone.[21] In addition, the combined Synthokine/rhG-CSF treatment sig-

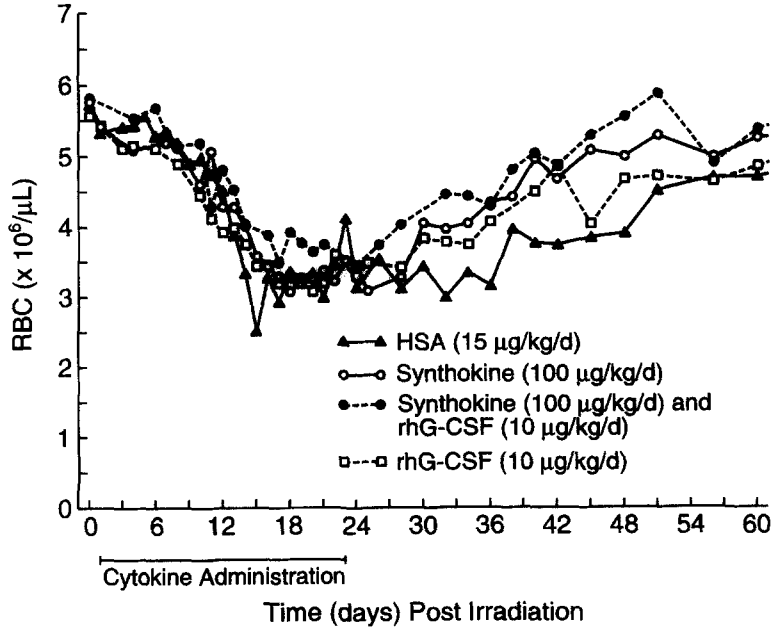

Fig 3. Effects of cytokine administration on peripheral blood RBCs in irradiated primates. The RBCs were observed in irradiated rhesus primates (700 cGy ^{60}Co) after Synthokine, G-CSF, Synthokine plus G-CSF or HSA after administration as described in the Materials and Methods. Data represent the mean of the RBC for the cytokine- or HSA-treated animals.

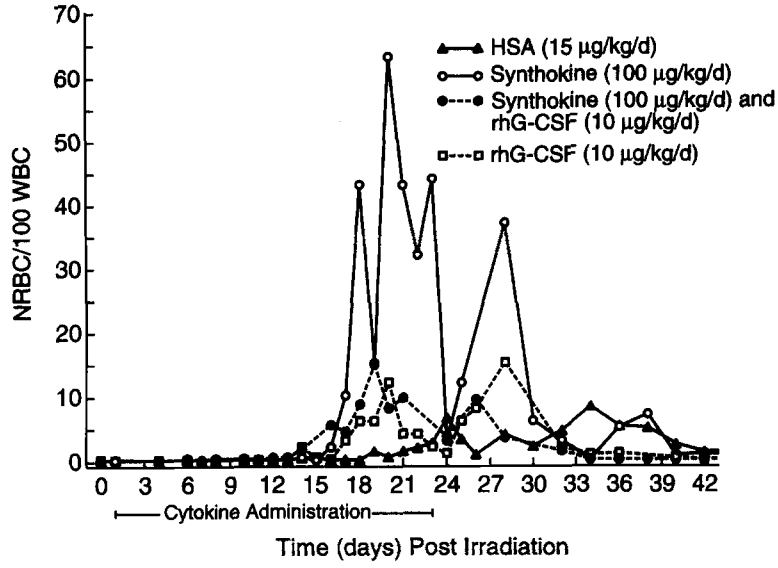

Fig 4. Effects of cytokine administration on peripheral blood NRBC in irradiated primates. The NRBC were observed in irradiated rhesus primates (700 cGy ^{60}Co) after Synthokine, G-CSF, Synthokine plus G-CSF, or HSA administration as described in the Materials and Methods. Data represent the mean of the NRBC for the cytokine- or HSA-treated animals.

nificantly decreased the platelet nadir versus Synthokine alone.

The Synthokine/rhG-CSF combination significantly reduced the duration of neutropenia versus HSA control or Synthokine alone-treated cohorts and further reduced, although not significantly, the neutropenic duration consequent to rhG-CSF administration. It is of interest that both the Synthokine alone and the combination Synthokine/rhG-CSF treatment significantly reduced the neutrophil nadir associated with HSA control or rhG-CSF administration. rhG-CSF did not significantly modify the neutrophil nadir associated with this dose of irradiation.

Various combinations of cytokines have been evaluated in nonhuman primate models of radiation- or drug-induced myelosuppression. Those reported include IL-3/GM-CSF, IL-3/IL-6 , IL-6/G-CSF, IL-6/GM-CSF, and the fusion protein PIXY-321.[2-4, 6, 8-10] To these, we add the Synthokine/G-CSF combination reported herein. Of these protocols, the most efficacious combination for production of both cell lineages, platelets, and neutrophils, relevant for early reconstitution within their respective models, has been Synthokine/G-CSF, IL-3/GM-CSF in concomitant protocols, and PIXY-321.[2-4] Each of the other combinations showed efficacy in either one lineage (IL-3/IL-6) or, if both lineages were increased (IL-6/G-CSF, IL-6/GM-CSF), the responses were not significantly greater than the respective cytokines used alone.[6,8-10] The combination of IL-3/IL-6 in sequential protocol only significantly reduced the thrombocytopenic duration relative to HSA controls and concomitant IL-3/IL-6 but not IL-6 or IL-3 alone.[9] Neutrophil recovery was unaffected by the concomitant or sequential IL-3/IL-6 protocol or with IL-3 and IL-6 as monotherapy. The concomitant IL-6/G-CSF or IL-6/GM-CSF combinations, although increasing both platelets and neutrophils each affected their respective lineages in a fashion not significantly different than either cytokine alone.[6,8] The efficacy of combined IL-3/GM-CSF was also found to be protocol or schedule dependent but somewhat different from the above-mentioned IL-3/IL-6 combination. Coadministration of IL-3/GM-CSF was significantly better in reducing both the durations of neutropenia and thrombocytopenia relative to the sequential IL-3/GM-CSF protocol or IL-3 and GM-CSF as monotherapy.[4] In this regard, the IL-3/GM-CSF fusion protein, PIXY-321, was also shown to promote earlier regeneration of both platelets and neutrophils.[2,3]

Previous in vitro studies had shown the augmentation of bone marrow-derived myeloid and megakaryocytic colony formation and splenic-derived blast cell colony formation when IL-3 was added to G-CSF.[14-17] More recently, Aglietta et al[20] showed the in vivo priming of IL-3 on bone marrow progenitor cells for subsequent ex vivo stimulation by lineage-specific later-acting cytokines such as G-CSF and GM-CSF. In addition to the noted priming effect, IL-3 sustains the viability of bone marrow cells and hematopoietic progenitor cells, and, in concordance, IL-3 receptors are found to be expressed on multipotent as well as on lineage-committed progenitor cells in rhesus monkey CD34$^+$ marrow cells.[24-28] These results suggest that the increase in mature cell lineages is dependent on the presence of Synthokine-primed, G-CSF–sensitive progenitor cells. In this regard, the Synthokine IL-3 receptor agonist has been shown to possess significantly greater hematopoietic activity on human marrow-derived progenitors with equivalent, inflammatory activity relative to native IL-3.[22] The responses noted herein may be an underestimate of the in vivo Synthokine activity, because it is known that native IL-3 and Synthokine have only 81% and 64%, respective, homology to rhesus IL-3 (unpublished results). Also, van Gils et al[28] have shown that native human IL-3 does not bind efficiently to rhesus marrow derived-progenitor cells.

The recent cloning and production of the c-mpl ligand and the identification of a new group of multifunctional agonists of the human IL-3 and G-CSF (Myelopoietin) or c-mpl receptor (Promegapoietin) complexes have provided another group of molecules with the potential to ameliorate radiation or drug-induced thrombocytopenia.[29-36] Administration of c-

mpl ligand to normal rodents and primates has shown prompt lineage-specific production of platelets associated with megakaryocyte progenitor cell expansion and megakaryocyte maturation.[29-31,37-40] The preclinical therapeutic efficacy of the c-mpl ligand has recently been shown in both rodent and nonhuman primate models of moderate and severe radiation or chemotherapy-induced thrombocytopenia.[37,38,41-46] The combination of c-mpl ligand with G-CSF is currently being evaluated in several models and the suspected dual-lineage specificity in therapeutic benefit appears to prevail.[38,42,43,46] It is of interest that a multifunctional human IL-3/G-CSF receptor agonist, Myelopoietin, stimulates regeneration of both neutrophils and platelets in a manner comparable to that noted for combined c-mpl ligand and G-CSF.[38,42,43,46,47]

Combined Synthokine/G-CSF therapy clearly enhanced hematopoietic regeneration as evidenced by improved neutrophil and platelet nadirs and decreased durations of both neutropenia and thrombocytopenia while maintaining transfusion independence. The demonstrated therapeutic efficacy of combined Synthokine and rhG-CSF in a concomitant protocol suggests clinical efficacy of this combination for treatment of thrombocytopenia and neutropenia in the myelosuppressed patient.

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